# Dendritic Epidermal T Cells: Activation Requirements and Phenotypic Characterization of Proliferating Cells

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Dendritic epidermal T cells (DETC) are CD45$^+$, Thy-1$^+$, CD5$^-$, CD8$^-$, CD4$^-$ murine lymphocytes that express surface-bound CD3 antigens associated with T cell receptor $\gamma/\delta$ heterodimers. Using epidermal cells greatly enriched for DETC and depleted of Langerhans cells, we found that DETC have growth requirements quite different from those of accessory cell-depleted lymph node and splenic T cells. Although the latter cells strongly proliferate in response to phorbol myristate acetate (PMA) + ionomycin, DETC, when exposed to interleukin-1 (IL-1), interleukin-3 (IL-3), concanavalin A (ConA), PMA, and ionomycin used either alone or in combination, do not exhibit significant mitotic activity. Recombinant interleukin 2 (rIL-2), albeit ineffective by itself, leads to vigorous proliferation of DETC when used with either ConA or PMA + ionomycin + IL-1. In contrast, the combination of PMA and recombinant interleukin-4 (rIL-4), which triggers growth of lymph node T cells, does not induce proliferation of DETC. Although a portion of proliferating DETC expressed CD8 antigens, essentially none bore detectable amounts of surface-bound CD4 or CD5 antigens, or both. Continuing stimulation of primary DETC cultures with lectin/lymphokine-rich media results in the propagation of cells with the essential phenotypic features of resident DETC. *J Invest Dermatol 92:763–768, 1989*

In 1983, the authors and others [1,2] described a hitherto unrecognized cell system within the murine epidermis. These cells are bone marrow-derived [1,3–5], display a highly dendritic morphology in situ, and bear large amounts of the Thy-1 alloantigen on their surface [1,2]. They are clearly distinct from all other epidermal cells (EC), i.e., keratinocytes (KC), Langerhans cells (LC), and melanocytes, and have been referred to as Thy-1$^+$ dendritic epidermal cells (Thy-1$^+$DEC) [1,2,5,6]. In single EC suspensions, these cells exhibit a round shape and comprise only 1–2% of all EC [1,2].

Although Thy-1$^+$DEC are different from most peripheral T cells as evidenced by their lack of CD5, CD4, and CD8 antigens [6], compelling evidence exists that they belong to the T cell system: they are numerically reduced in athymic mice [1]; they proliferate in response to concanavalin A (ConA) + interleukin 2 (IL-2) [7]; and they uniformly express surface-bound CD3 antigens [8]. These CD3 antigens are predominantly, if not exclusively, associated with disulfide-linked 35 kD/45 kD T cell receptor (TCR) $\gamma/\delta$ heterodimers [9].

In view of the presence of TCR on the surface of Thy-1$^+$DEC, we have recently proposed that these cells should be renamed dendritic epidermal T cells (DETC) [9]. Cells with similar TCR configurations have been described on a subset of thymocytes [10–13], peripheral T cells [14–17], and T cell leukemias [18] in humans and mice. It appears that, as opposed to lymphoid organs, the adult murine epidermis represents a tissue that contains a homogeneous population of CD3$^+$, TCR $\gamma/\delta^+$, TCR $\alpha/\beta^-$ cells (i.e., DETC), but is devoid of any other lymphoid elements [8, 9]. We, therefore, felt that the investigation of activation, growth, and possibly differentiation requirements of these cells might not only provide a clue as to the functional capacity of DETC, but may also be representative of the entire system of CD3$^+$, TCR $\gamma/\delta^+$, CD5$^-$, CD4$^-$, CD8$^-$ lymphocytes.

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Abbreviations:

BSA: bovine serum albumin
CAS: concanavalin A-conditioned rat spleen cell supernatant
ConA: concanavalin A
DETC: dendritic epidermal T cells (previously termed Thy-1$^+$DEC)
EC: Epidermal cells
FACS: fluorescence activated cell sorter
FBS: fetal bovine serum
FITC: fluorescein-isothiocyanate
$^3$H-TdR: [$^3$H]thymidine
rIL-1: recombinant interleukin 1
rIL-2: recombinant interleukin 2
IL-2: interleukin 2
IL-3: interleukin 3
rIL-4: recombinant interleukin 4
KC: Keratinocytes
LC: Langerhans cells
LNT: Lymph node T cells
$NaN_3$: sodium azide
PBS: phosphate-buffered saline
PMA: phorbol myristate acetate
ST: Splenic T cells
TCR: T cell receptors
Thy-1$^+$DEC: Thy-1 positive dendritic epidermal cells

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## MATERIALS AND METHODS

**Animals** Male C3H/He mice and male Lewis rats were obtained from the Versuchstierzucht und—haltung der Universität Wien, Himberg, Austria.

**First Step Antibodies** Information provided in Table I includes antibody specificities and the concentrations of the antibodies that were used for the different procedures.

**FACS Analysis and Conventional Immunofluorescence Microscopy** The various monoclonal antibodies (Table I) [19–21] and isotype-matched control reagents were diluted in phosphate-buffered saline (PBS) containing 2% bovine serum albumin (BSA) and 0.1% sodium azide ($NaN_3$) to the respective working dilutions. Cells ($10^5$) were incubated in 200 $\mu$l of the diluted first antibody for 30 min on ice and washed three times with PBS/2% BSA/0.1% $NaN_3$ before incubation with the appropriate fluorescein (FITC)-labeled second antibodies. They included FITC-mouse anti-rat Ig kappa light chain (clone 18.5; mouse $IgG_{2a}$) [22], FITC-F(ab')$_2$ goat anti-mouse Ig (Grub antibodies, Scandic, Vienna, Austria), and FITC-F(ab')$_2$ goat anti-hamster Ig (Cappel Labs., Cochranville, PA). Stained cells were either analyzed by conventional fluorescence microscopy (Leitz Ortholux II; Wetzlar, F.R.G.) or on a fluorescence activated cell sorter (FACS 440; Becton Dickinson, Mountain View, CA).

**Cell Preparations** Single EC suspensions were prepared from mouse ear skin by standard trypsinization procedures as described previously [23]. All preparation steps, except trypsinization, were performed in RPMI 1640 containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 50 $\mu$g/ml gentamycin (all Flow Laboratories, Irvine, UK).

Keratinocytes were prepared by treatment of unfractionated EC suspensions with anti-$Ia^k$ and anti-Thy-1.2 monoclonal antibodies (working dilution, Table I) for 30 min at 0°C followed by complement (C'; Cedarlane, Hornsby, Ontario, Canada) for 60 min at 37°C. Dead cells were then removed by Lympholyte-M (Cedarlane; optical density 1.087) density gradient centrifugation for 10 min at 1200 rpm.

For enrichment of DETC and LC from EC suspensions, single EC ($2 \times 10^8$) were passed over a discontinuous Percoll gradient (Pharmacia, Uppsala, Sweden; Percoll/RPMI 1640 containing 0.25 M sucrose) consisting of a 20% and 40% fraction of 15 ml each, in 50-ml Falcon tubes (Becton Dickinson, Oxnard, CA). After centrifugation at 1200 rpm for 10 min, cells of the interface between the Percoll fractions were recollected and treated with anti-Thy-1.2 + C'. Cells in the pellet were first subjected to a 1-min osmotic shock treatment with distilled water, which resulted in lysis of more than 95% of the EC (predominantly differentiating KC). Subsequently, they were either treated with anti-$Ia^k$ + C' alone or with both anti-Lyt-1.1 and anti-$Ia^k$ + C'. Both the original interface and the pellet fraction were then freed of dead cells by centrifugation over Lympholyte-M. Cells dervied from the interface fraction were substantially enriched for $CD45^+$, $Ia^+$, Thy-$1.2^-$ cells (Fig 1 A,B,C). This cell population will be further referred to as LC (the extent of enrichment ranged between 35%–70%). Cells from the pellet fraction were greatly enriched for $CD45^+$, Thy-$1.2^+$, $Ia^-$ cells (Fig 1 D,E,F). This cell population contained large numbers of $CD3^+$ cells, but no $CD5^+$, $CD4^+$, or $CD8^+$ cells (data not shown) and will be further referred to as DETC (the quality of enrichment ranged between 60%–90%). The viability of the EC suspensions and their subpopulations was consistently >95% as assessed by trypan blue exclusion.

Purified peripheral T cells were prepared by passing unseparated lymph node or spleen cells through a nylon wool column. Nonadherent effluent cells were subsequently treated with anti-$Ia^k$ + C'. These cells were designated lymph node T cells (LNT) and splenic T cells (ST), respectively. Their viability was always >99%.

**Producton of Lymphokine-rich ConA-activated Rat Spleen Cell Supernatants (CAS)** Lewis rats were killed by ether anesthesia, their spleens were removed, and single cell suspensions were

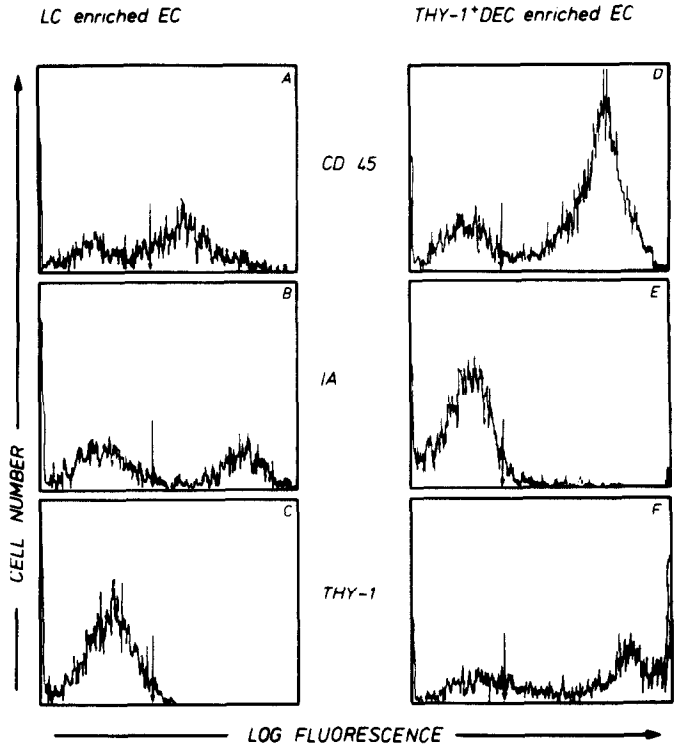


**Figure 1.** Immunophenotypic features of LC-enriched EC (=LC) and Thy-$1^+$DEC-enriched EC (=DETC). LC-enriched EC *(left panel)* and Thy-$1^+$DEC-enriched EC *(right panel)* were reacted with anti-CD45, anti-$Ia^k$, and anti-Thy-1.2 monoclonal antibodies, respectively, using an indirect immunofluorescence procedure, and were then analyzed by flow cytometry. Dead cells were excluded by propidium iodide staining; *arrows* denote the position of the cut-off channel fixed with the negative control. LC-enriched EC are Thy-$1^-$ (3%;C), but many of them bear CD45 (68%;A) and Ia antigens (42%;B). In contrast, Thy-$1^+$DEC-enriched EC are $Ia^-$ (5%;E), but most of them bear CD45 (87%;D) and Thy-1 (90%;F) antigens.

**Table I.** Antibodies Used for Immunofluorescence and Cytotoxicity

| Antibody | Source[a] | Working Dilution: Staining | Working Dilution: Cytotoxicity |
|---|---|---|---|
| Mouse anti-mouse Thy-1.2 (IgM, ascites) | NEN | 1:300 | 1:10,000 |
| Rat anti-mouse Thy-1.2 ($IgG_{2b}$, purified antibody) | BD | 1:100 | — |
| Mouse anti-mouse Lyt-1.1 (CD5) ($IgG_{2b}$, ascites) | NEN | — | 1:10,000 |
| Rat anti-mouse Lyt-1 (CD5) ($IgG_{2a}$, purified antibody) | BD | 1:100 | — |
| Rat anti-mouse Lyt-2 (CD8) ($IgG_{2a}$, purified antibody) | BD | 1:100 | — |
| Rat anti-mouse L3T4 (CD4) ($IgG_{2b}$, clone GK1.5, ascites) | EMS | 1:100 | — |
| Mouse anti-mouse $Ia^k$ ($IgG_{2b}$, purified antibody) | BD | 1:100 | 1:100 |
| Mouse anti-mouse Ly-5.1 (CD45) ($IgG_{2a}$, ascites) | NEN | 1:100 | — |
| Rat anti-mouse IL-2 receptor (CD25) (IgM, clone 7D4, ascites) | EMS | 1:100 | — |
| Hamster anti-mouse CD3 (IgG, clone 145-2C11, culture supernatant) | JAB | neat | |

[a] NEN, New England Nuclear; BD, Becton Dickinson; Antibodies GK 1.5 [19] and 7D4 [20] were kindly provided by Dr. Ethan M. Shevach (EMS; NIAID, Bethesda, MD). Clone 145-2C11 [21] was a generous gift from Dr. Jeffrey A. Bluestone (JAB, NCI, Bethesda, MD).

prepared by mechanical agitation. After red cell lysis with ammonium-chloride buffer, cells were frequently washed in FBS-supplemented RPMI 1640 and adjusted to a concentration of $10 \times 10^6$/ml. They were then cultured for 36 h in RPMI 1640 containing 10% FBS, 2 mM L-glutamin, 50 μg/ml gentamycin, 100 U/ml penicillin (Flow Labs), 100 μg/ml streptomycin (Flow Labs), 0.25 μg/ml amphotericin (Flow Labs), 1 mM sodium pyruvate (Flow Labs), 0.1 mM nonessential amino acids (Flow Labs), 25 mM Hepes buffer (Flow Labs), $5 \times 10^{-5}$ M 2-mercaptoethanol (Gibco, Grand Island, NY), 10 μg/ml ConA (Pharmacia), and 1 μg/ml indomethacin (Sigma Chem. Corp., St. Louis, MO) in 5% $CO_2$/95% air. Thereafter, culture supernatants were collected by centrifugation of the cell suspension, sterile-filtered, and stored at −20°C until further use.

**Cell Proliferation Assays and Growth Factors** Epidermal cells, LC, KC, DETC, LNT, or ST ($5 \times 10^3$, $5 \times 10^4$/well) were cultured in 96 well round-bottom microtiter plates (Flow Labs), in the presence or absence of one or more of the following agents (depicted in final concentrations): Con A (2.5 or 5 μg/ml), phorbol myristate acetate (PMA; Sigma; 10 ng/ml), ionomycin (Calbiochem; La Jolla, CA; 500 ng/ml), recombinant human interleukin-1α (rIL-1; Genzyme Corp., Boston, MA; 5 U/ml), recombinant human interleukin-2 (rIL-2; Amgen Biol., Thousand Oaks, CA; 50 or 100 U/ml), recombinant murine interleukin-4 (rIL-4; Genzyme Corp.; 200 U/ml), purified murine interleukin-3 (IL-3; Genzyme Corp.; 20 U/ml), 10% or 20% CAS. Culture medium consisted of RPMI 1640 containing 10% FBS, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 25 mM Hepes buffer, 50 μg/ml gentamycin, $5 \times 10^{-5}$ M 2-mercaptoethanol, and 1 μg/ml indomethacin. Triplicate cultures were incubated at 37°C in 5% $CO_2$ for 4–5 d (inspection of the cultures had revealed significant cell growth on these days). Proliferation was assessed by [$^3$H]thymidine uptake (1 μCi $^3$H-TdR/well; NEN, Boston, MA) during the last 12 h of the culture period. The data are expressed as mean cpm of triplicate samples.

## RESULTS

**DETC Are the Only Epidermal Cells To Proliferate in Response to ConA + rIL-2** It has been previously reported that DETC-enriched EC proliferate vigorously to ConA + IL-2, whereas DETC-depleted EC fail to do so [7]. Using a different enrichment procedure, we have obtained similar results, which are depicted in Table II. Using $5 \times 10^4$ cells/well, we observed in three individual experiments that unseparated EC (column 1) undergo marked proliferation in response to ConA and rIL-2, but only weak proliferation when stimulated with either ConA (5 μg) or CAS alone. No proliferation was observed with rIL-2. All these agents failed to induce proliferation above background in KC and LC (columns 2, 3)*. DETC showed vigorous proliferation to ConA + rIL-2 and, to a lesser extent, in response to ConA alone and to CAS (column 4). These data demonstrate that 1) the capacity of EC to proliferate in response to ConA + rIL-2 resides within a CD45$^+$, Thy-1$^+$, Ia$^-$ cell population, most likely DETC and 2) that Ia-bearing LC are not required for the occurrence of this event.

**Table II.** Proliferative Response of EC, LC, KC, and DETC to ConA, rIL-2, and CAS: Representative Experiment

| Supplements Added | Responding Cell Populations[a] [$^3$H]Thymidine Uptake, cpm | | | |
|---|---|---|---|---|
| | EC | KC | LC[c] | DETC[d] |
| Medium | 118[e] | 3,714 | 1,371 | 1,098 |
| ConA (5 μg) | 2,430 | 2,964 | 882 | 20,514 |
| ConA (2.5 μg) | 880 | NT[b] | 1,041 | 6,610 |
| ConA (2.5 μg) + rIL-2 (50 U) | 9,023 | 2,220 | 1,496 | 39,045 |
| rIL-2 (50 U) | 970 | 3,433 | 488 | 1,545 |
| CAS 10% | 1,559 | 5,062 | 1,843 | 17,644 |

[a] The different cell populations were seeded in 96 well round-bottom microtiter plates at a density of $5 \times 10^4$ cells/well.
[b] NT = not tested.
[c] Contained 42% Ia$^+$ cells.
[d] Contained 90% Thy-1$^+$ cells.
[e] SEM were regularly <15% and are omitted for clarity.

**Table III.** Comparison of DETC and ST in Their Proliferative Response to rIL-1, rIL-2, IL-3, and ConA

| Supplements Added | Responding Cell Populations[a] [$^3$H]Thymidine Uptake, cpm | | |
|---|---|---|---|
| | Experiment 1 | | Experiment 2 |
| | DETC[b] | ST | DETC[c] |
| ConA (5 μg) | 890[d] | 3,111 | 1,576 |
| ConA (2.5 μg) | 115 | 179 | 1,277 |
| ConA (2.5 μg) + rIL-1 (5 U) | 283 | 1,796 | 2,589 |
| ConA (2.5 μg) + rIL-2 (100 U) | 26,071 | 86,731 | 14,102 |
| ConA (2.5 μg) + IL-3 (20 U) | 840 | 1,634 | 2,473 |
| ConA (2.5 μg) + rIL-1 (5 U) + rIL-2 (100 U) | 28,838 | 55,529 | 15,723 |
| ConA (2.5 μg) + rIL-1 (5 U) + IL-3 (20 U) | 506 | 3,951 | 5,689 |
| ConA (2.5 μg) + rIL-2 (100 U) + IL-3 (20 U) | 26,163 | 98,058 | 14,725 |
| rIL-1 (5 U) | 310 | 992 | 1,994 |
| rIL-2 (100 U) | 511 | 843 | 2,022 |
| IL-3 (20 U) | 277 | 4,125 | 1,858 |
| rIL-1 (5 U) + rIL-2 (100 U) | 677 | 718 | 2,562 |
| rIL-1 (5 U) + IL-3 (20 U) | 286 | 2,802 | 2,069 |
| rIL-2 (100 U) + IL-3 (20 U) | 327 | 2,222 | 2,916 |
| rIL-1 (5 U) + rIL-2 (100 U) + IL-3 (20 U) | 630 | 733 | 2,706 |
| Medium | 186 | 89 | 1,575 |

[a] Cells were seeded in 96 well round-bottom microtiter plates at a density of $5 \times 10^3$ cells/well.
[b] Containing 86% Thy-1$^+$ cells.
[c] Containing 67% Thy-1$^+$ cells.
[d] SEM were regularly <15% and are omitted for clarity.

**Activation and Growth Factor Requirements of DETC as Compared With ST and LNT†** Because even minute numbers of passenger T cells possibly present in the DETC-enriched cell population, which might lead to erroneous conclusions about the actual activation requirements of DETC, we used $5 \times 10^3$ instead of $5 \times 10^4$ cells/well, and in certain experiments, the enrichment procedure for DETC included the use of both anti-Ia$^k$ + C′ and anti-Lyt-1.1 + C′.

As demonstrated in Table III, ConA + rIL-2 induced vigorous proliferative activity in both DETC and ST. ConA or rIL-2 alone, as well as a combined use of ConA and either rIL-1 or IL-3, or both, and combinations of the three cytokines in the absence of ConA, were essentially incapable of stimulating proliferation of either DETC or ST.

In a second series of experiments, we compared DETC to LNT and ST in their potential to respond to PMA and ionomycin, agents known to bypass the lectin-dependent pathway of lymphocyte activation [24]. As opposed to ST (Table IV, column 2), which strongly

* The enrichment procedure used for the various EC subpopulations cannot only be used for functional assays of DETC: the LC fraction led to a strong proliferative response of purified, allogeneic T cells, and KC readily attached to and spread on plastic surfaces (data not shown). The comparatively high background proliferation of KC (column 2) may possibly be due to a selective enrichment of those KC that are capable of autocrine growth factor production.

† As can be seen in Tables III, IV, and V, there is a certain variation between the single experiments in the magnitude, but not in the pattern, of the proliferative activity of DETC induced by the various stimuli. The reasons for this variation include 1) the tedious, multistep procedure for DETC-enrichment, 2) inevitable variations in cell number/well due to the multiple dilution steps of the stock suspension, and 3) differences in the quality of DETC enrichment (60%–90%).

proliferated in response to both PMA + ionomycin and to PMA + rIL-2, DETC (Table IV, columns 1, 3) failed to do so. The combination of PMA + ionomycin + rIL-2 induced only minimal growth in DETC, but stimulated ST to a maximal extent. It also appears that IL-1 may be an ancillary signal in the activation of DETC in that the addition of this cytokine greatly increases the proliferative response of DETC to PMA + ionomycin + rIL-2 (Table IV, columns 1,3). Furthermore, even the proliferation of DETC to ConA + rIL-2 is regularly enhanced by the addition of rIL-1 (Table IV, columns 1, 3). These data show that, upon costimulation with either ConA or PMA + ionomycin + rIL-1, IL-2 is an effective means to induce a strong proliferative response in DETC.

It has been reported recently that there exists an alternative growth factor to IL-2 involved in the proliferation of both T cell precursors and certain peripheral T cells in that the combined use of rIL-4 and PMA results in strong proliferation of day 14–15 fetal thymocytes [25,26], PNA-negative adult thymocytes [26], and accessory cell-depleted lymph node T cells [27]. We also observed that PMA + rIL-4, but not either substance alone, triggered marked proliferation in LNT but, in repeated experiments, consistently failed to stimulate a growth response in DETC (Table V).

**Table IV.** Proliferative Response of DETC and ST to Various Stimuli

| Supplements Added[a] | Responding Cell Populations[b] [$^3$H]Thymidine Uptake, cpm | | |
|---|---|---|---|
| | Experiment 1 | | Experiment 2 |
| | DETC[c] | ST | DETC[d] |
| ConA (2.5 μg) + rIL-1 (5 U) | 456[e] | 1,319 | 865 |
| ConA (2.5 μg) + rIL-2 (100 U) | 22,218 | 75,533 | 14,306 |
| ConA (2.5 μg) + PMA (10 ng) | 576 | 1,953 | 243 |
| ConA (2.5 μg) + ionomycin (500 ng) | 306 | 278 | 316 |
| ConA (2.5 μg) + PMA (10 ng) + ionomycin (500 ng) | 803 | 131,181 | 658 |
| ConA (2.5 μg) + rIL-1 (5 U) + rIL-2 (100 U) | 25,061 | 79,860 | 17,563 |
| ConA (2.5 μg) + rIL-1 (5 U) + PMA (10 ng) | 715 | 3,037 | 407 |
| ConA (2.5 μg) + rIL-1 (5 U) + PMA (10 ng) + ionomycin (500 ng) | 574 | 162,166 | 336 |
| ConA (2.5 μg) + rIL-2 (100 U) + PMA (10 ng) | 8,782 | 135,678 | 6,405 |
| ConA (2.5 μg) + rIL-2 (100 U) + ionomycin (500 U) | 22,325 | 26,431 | 18,530 |
| rIL-1 (5 U) + rIL-2 (100 U) | 964 | 708 | 1,373 |
| rIL-1 (5 U) + PMA (10 ng) | 392 | 1,211 | 746 |
| rIL-1 (5 U) + ionomycin (500 ng) | 296 | 84 | 288 |
| rIL-1 (5 U) + PMA (10 ng) + ionomycin (500 ng) | 428 | 100,889 | 866 |
| rIL-1 (5 U) + rIL-2 (100 U) + PMA (10 ng) | 280 | 62,837 | 1,408 |
| rIL-1 (5 U) + rIL-2 (100 U) + ionomycin (500 ng) | 1,660 | 5,152 | 2,123 |
| rIL-1 (5 U) + rIL-2 (100 U) + PMA (10 ng) + ionomycin (500 ng) | 7,922 | 142,691 | 4,806 |
| rIL-2 (100 U) + PMA (10 ng) | 497 | 30,022 | 676 |
| rIL-2 (100 U) + ionomycin (500 ng) | 465 | 2,868 | 441 |
| rIL-2 (100 U) + PMA (10 ng) + ionomycin (500 ng) | 2,560 | 160,991 | 2,117 |
| PMA (10 ng) + ionomycin (500 ng) | 229 | 142,590 | 469 |
| Medium | 276 | 568 | 273 |

[a] cpm obtained after addition of either ConA (2.5 μg), rIL-1 (5 U), rIL-2 (100 U), PMA (10 ng), or ionomycin (500 ng) were consistently < 700 in the case of DETC and < 1400 in the case of ST.
[b] Cells were seeded at 5 × $10^3$ cells/well.
[c] Contained 77% Thy-1$^+$ cells.
[d] Contained 65% Thy-1$^+$ cells.
[e] SEM were regularly < 15% and are omitted for clarity.

**Table V.** DETC Fail To Proliferate to PMA and rIL-4

| Supplements Added | Responding Cell Populations[a] [$^3$H]Thymidine Uptake, cpm | | |
|---|---|---|---|
| | Experiment 1 | | Experiment 2 |
| | DETC[b] | LNT | DETC[c] |
| rIL-1 (5 U) | 120[d] | 70 | 1,395 |
| rIL-2 (100 U) | 810 | 1,612 | 2,103 |
| rIL-4 (200 U) | 411 | 85 | 2,329 |
| ConA (2.5 μg) | 193 | 41 | 301 |
| ConA (2.5 μg) + rIL-1 (5 U) | 175 | 73 | 833 |
| ConA (2.5 μg) + rIL-2 (100 U) | 39,074 | 3,791 | 61,845 |
| ConA (2.5 μg) + rIL-4 (200 U) | 294 | 1,750 | 3,427 |
| PMA (10 ng) | 577 | 1,114 | 2,289 |
| PMA (10 ng) + rIL-1 (5 U) | 372 | 980 | 1,250 |
| PMA (10 ng) + rIL-2 (100 U) | 1,039 | 28,328 | 1,898 |
| PMA (10 ng) + rIL-4 (200 U) | 604 | 7,946 | 1,335 |
| PMA (10 ng) + rIL-1 (5 U) + rIL-4 (200 U) | 846 | 10,069 | 1,490 |
| PMA (10 ng) + rIL-2 (100 U) + rIL-4 (200 U) | 2,511 | 49,444 | 2,486 |
| Medium | 283 | 59 | 385 |

[a] Cells were seeded at 5 × $10^3$ cells/well.
[b] Contained 86% Thy-1$^+$ cells.
[c] Contained 90% Thy-1$^+$ cells.
[d] SEM were regularly < 15% and are omitted for clarity.

**Phenotypic Analysis of Proliferating DETC** Phenotypic screening of either DETC or ST upon stimulation with ConA (2.5 μg/ml) + rIL-2 (100 U/ml) was performed 6 d after the initiation of the cultures. FACS analysis (Fig 2*A*) revealed that the vast majority of proliferating DETC were strongly Thy-1$^+$ and CD45$^+$. Although the search for CD5 and CD4 antigens yielded negative results, a varying percentage (ranging from 0%–24%) of cells expressed surface-bound CD8 antigens in three different experiments. This phenotypic profile is profoundly different from that of ST, which predominantly contained CD5$^+$ cells (Fig 2*B*).

In the presence, but not in the absence, of CAS (20%)-containing media, vigorous proliferation of DETC can be maintained in the absence of feeder cells or Ia$^+$ accessory cells, or both, for prolonged

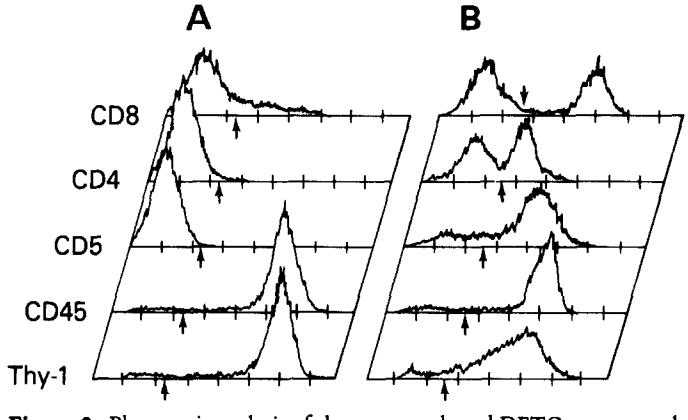


**Figure 2.** Phenotypic analysis of short-term cultured DETC as compared to ST. After 6 d of continuous stimulation with ConA and rIL-2, cells were reacted with a panel of monoclonal antibodies using an indirect immunofluorescence procedure and analyzed by flow cytometry. Dead cells were excluded by propidium iodide staining. *Arrows* denote the position of the cut-off channel fixed with the negative controls. DETC (*A*) are strongly Thy-1$^+$ and CD45$^+$, but completely lack CD4 and CD5 antigens. CD8 antigens are present on a small subpopulation of proliferating cells (~ 24%). In contrast, the vast majority (85%) of proliferating ST (*B*) express CD5 antigens; CD4$^+$ and CD8$^+$ antigens were detected on 46% and 51% of these cells, respectively.

periods of time (Tschachler et al, in preparation) [28]. As opposed to short-term stimulated DETC that contained a variable portion of $CD8^+$ cells (see above), culture of these cells for an additional 6 wk consistently resulted in a uniform phenotypic profile: $CD45^+$, Thy-$1^+$, $CD3^+$, $CD5^-$, $CD4^-$, $CD8^-$, and $Ia^-$ (Fig 3). Thus, long-term cultured DETC phenotypically resemble their in situ counterpart with the notable exception that cultured DETC express IL-2 receptors as defined by anti-CD25 reactivity (Fig 3).

## DISCUSSION

In this study, we have demonstrated that DETC, which comprise only 1%–2% of murine EC, can be greatly enriched for (60%–90%) by a combined use of density gradient centrifugation techniques and removal of other EC populations by osmotic and antibody-dependent complement lysis. These DETC-enriched EC were used to study the activation and growth-promoting requirements of DETC. Delivering a variety of T cell activating signals, we found 1) that activation requirements of DETC are different from those of peripheral T lymphocytes and 2) that, upon continuous stimulation with lectin/lymphokine-rich media, a selective propagation of cells with the essential phenotypic features of DETC can be achieved.

The most prominent difference in activation and growth requirements between DETC and LNT/ST is that, in the absence of T cell growth factors, exposure of DETC to a variety of stimuli (rIL-1, ConA, PMA, ionomycin) used either alone or in combination never results in a reliable growth response. In sharp contrast, PMA + ionomycin induces strong proliferation of LNT and ST. Nevertheless, it appears that both ConA and PMA + ionomycin provide an important activation signal for DETC in that, upon addition of rIL-2, these cells undergo proliferative activity. Whereas ConA + rIL-2 was the most effective stimulus for proliferation, PMA + ionomycin + rIL-2 led only to marginal mitotic activity of DETC. Repeated experiments showed, however, that the latter response could be considerably enhanced by the addition of rIL-1. In fact, PMA + ionomycin + rIL-1 was the only stimulus that together with rIL-2 was effective in bypassing the otherwise obligatory need for ConA + IL-2 in growth induction of DETC. This finding can be interpreted in several ways. First, it may well be that PMA + ionomycin alone render DETC IL-2 responsive to a limited extent and that the function of rIL-1, albeit ineffective by itself, is to enhance this effect. On the other hand, one should not forget that EC even when greatly enriched for DETC still contain considerable numbers of KC known to be capable of IL-1 production [29]. It is thus conceivable that the slight proliferation seemingly induced by PMA + ionomycin + rIL-2 alone must be attributed to a costimulating effect of KC-derived IL-1 that, in the case of high DETC enrichment, would be available in only limited quantities. Excess amounts of rIL-1 would then allow optimal proliferation. A similar mechanism may actually be operative in the activation of DETC-enriched EC by ConA + rIL-2: we have occasionally observed that the magnitude of the proliferative response induced by ConA + rIL-2 + rIL-1 was higher than that seen with ConA + rIL-2 alone.

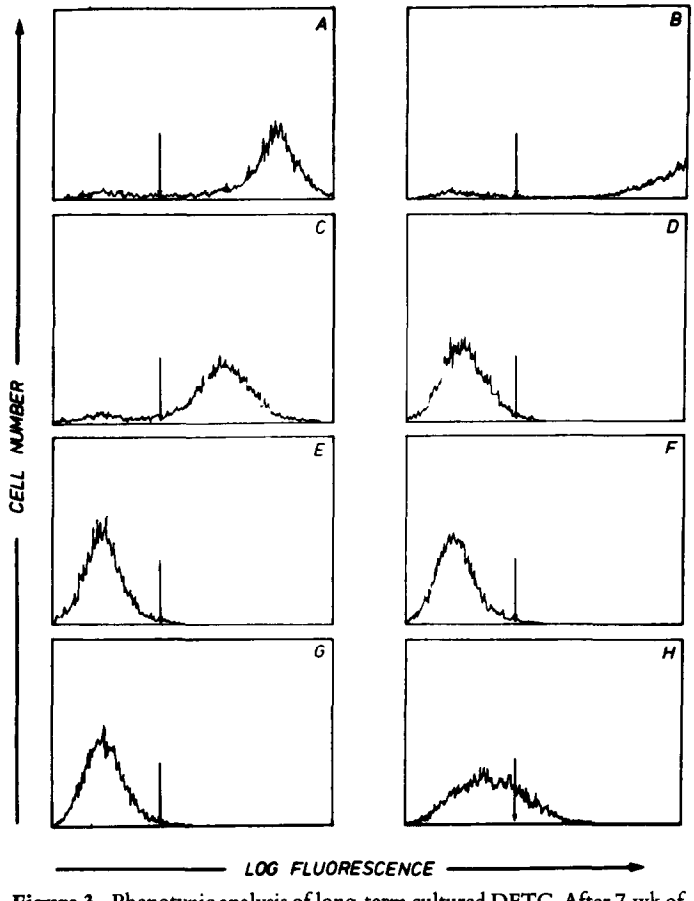


**Figure 3.** Phenotypic analysis of long-term cultured DETC. After 7 wk of continuous stimulation with CAS (20%)-containing media, cells were reacted with a panel of monoclonal antibodies using an indirect immunofluorescence procedure and analyzed by flow cytometry. Dead cells were excluded by propidium iodide staining. *Arrows* denote the position of the cut-off channel fixed with the negative controls. Virtually all cells are $CD45^+$ *(A)*, Thy-$1^+$ *(B)*, and $CD3^+$ *(C)*, but lack CD5 *(D)*, CD4 *(E)*, CD8 *(F)*, and Ia *(G)* antigens. A subpopulation of these cells (27%) bears CD25 antigens *(H)*.

Although resident DETC lack IL-2 receptors [6] and do not proliferate in response to rIL-2 alone, it appears that on delivery of appropriate costimulatory signals (ConA or PMA + ionomycin + IL-1), IL-2 rather than IL-4 is the critical growth factor for DETC. In this regard, DETC differ from $CD4^-$, $CD8^-$ (double-negative) fetal and adult thymocytes that, upon activation with either ConA or PMA, proliferate in response to both growth factors even though the IL-4 response is significantly higher in magnitude than that seen with IL-2 [26]. Similarities or differences in activation and growth requirements between DETC and double-negative thymocytes [30,31] should, however, not be overinterpreted. Whereas DETC are a homogeneous population of $CD3^+$, TCR $\gamma/\delta^+$, $CD4^-$, $CD8^-$ lymphocytes [8,9], $CD4^-$, $CD8^-$ thymocytes exhibit a pronounced heterogeneity in TCR expression ($CD3^-$, $CD3^+$–TCR $\gamma/\delta^+$, $CD3^+$–TCR $\alpha/\beta^+$) [13, 32–34]. Despite the demonstration of IL-2 production by a ConA-stimulated DETC-derived cell line [35] and by a ConA- or anti-CD3 $\epsilon$-stimulated DETC-derived T cell hybridoma [36], it appears from this study that freshly isolated, activated (ConA or PMA + ionomycin + IL-1) DETC do not produce IL-2 in quantities sufficient to sustain autocrine growth. Actually, one wonders whether a putative activation of these cells in vivo (e.g., by antigens or by EC-derived cytokines) is indeed followed by their proliferation; it appears equally possible that physiologic activation of DETC is not reflected in their proliferation but only in the induction/generation of as yet undefined secretory and/or effector mechanisms.

Although the occurrence of CD5, CD8, and CD4 antigens has never been observed on resident DETC [6,9], it was interesting to see that a varying percentage of DETC proliferating in response to CAS-containing media expressed CD8 antigens. This, however, was a transitory phenomenon as, on continuous stimulation, these $CD8^+$ DETC gradually disappeared giving rise finally to cell lines displaying the essential features of resident Thy-$1^+$DEC, i.e., $CD45^+$, Thy-$1^+$, $CD3^+$, $CD5^-$, $CD8^-$, $CD4^-$, $Ia^-$ [28]. Interestingly, the gradual disappearance of $CD8^+$ DETC coincided with the progressive loss of KC "contaminating" freshly isolated DETC. Should this coincidence be more than fortuitous, it is conceivable that similar to the critical role of the thymic stroma for the maturation of thymic stem cells [37–39], KC, and possibly also LC, and their products may induce maturational events in DETC. The lack of further differentiation of DETC into either $CD4^+$ or $CD8^+$ cells would then be a consequence of the progressive loss of KC imposed by the culture conditions employed. Questions concerning the potential for differentiation of DETC and the environment necessary

for such a hypothetical event are currently under investigation in our laboratory.

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